A NEW TRANSFORMING AGENT DETERMINING PATTERN OF METABOLISM OF GLUCOSE AND LACTIC ACID IN *PNEUMOCOCCUS*[1]

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THE starting point of the present investigation was the observation that the Type III encapsulated pneumococci arising from transformations induced in certain unencapsulated strains are of two sorts: the majority give rise to colonies having diameters approximately 3 times larger than rough colonies have, while a few form colonies which are 4 times larger. Clones set up from the two sorts maintain indefinitely, under appropriate conditions, their characteristic difference in colony size. The experiments to be described below are concerned with the origin and nature of the pneumococci which give rise to large colonies (LC pneumococci).

The interest of the observation emerges from the following considerations. In the past it has been noted that the size of a pneumococcal colony is often correlated with the amount of capsular polysaccharide secreted (8, 9, 14). Thus, the finding of the LC clones raised the problem, already considered in an earlier publication (15), of whether the Type III capsular agent acts as a discrete unit, or whether, on the contrary, it induces the formation of a spectrum of transformed bacteria exhibiting varying degrees of polysaccharide secretion. If the latter were true, LC clones might be pneumococci which secrete very large amounts of capsular polysaccharide, and belong thus in the upper limits of such a spectrum. In the earlier study it was concluded that the capsular transforming agent acts in an all-or-none fashion. The present study confirms this conclusion, for it was found that the colonies of the transformed Type III pneumococci form two discrete distributions with respect to diameter. No evidence for a spectrum could be obtained.

The experiments to be described show that LC pneumococci are produced by a double transformation, in the course of which a bacterium acquires not only the capsular agent, but also a hitherto unrecognised agent, present in extracts prepared from the Type III strain usually employed in capsular transformation studies. The new agent (LC agent) is apparently independent

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of the capsular agent, both as a genetic entity and with respect to its physiological activities, for it can be acquired singly by unencapsulated bacteria, producing in them a change which results in their forming colonies which are slightly larger and distinctly more opaque than those of ordinary unencapsulated clones. A study of the metabolic differences between normal and LC strains will be described. The results of this study demonstrate that LC strains have a distinctive pattern of glucose metabolism, and that they fail to oxidize lactic acid.

The present report is subdivided into two chapters, one dealing with biometric and transformation studies, the other describing metabolic studies.

I. BIOMETRIC AND TRANSFORMATION STUDIES

Two questions were investigated: 1) Are capsular transformations induced in all-or-none fashion? and 2) What is the origin of the LC pneumococci? These problems were investigated by the measurement of many colonies of encapsulated pneumococci, obtained by spreading on petri dishes populations of unencapsulated pneumococci in which transformations had been induced by treatment with various specific transforming extracts.

MATERIAL AND METHODS

The methods of culture, preparation of transforming agents, and induction of transformations were those described in a previous publication (4). The only modification introduced was the more careful standardization of the blood-agar plates on which the colonies to be measured were grown. The important factors to control are glucose content and surface humidity. With respect to the former, 2.0 cc of a sterile 2.5 per cent glucose solution were added for every 150 cc of agar medium. Surface humidity was reduced by drying the plates overnight at 37° C. In any single experiment the same volume of culture, or dilution thereof, was spread on all of the plates. In the early part of the work, sterile defibrinated rabbit blood was employed in the agar medium, while later horse blood was used. Mr. Lemetayer, Director of the Institut Pasteur at Garches, and Mr. Girard, Chef des Services de Saignées, very kindly supplied horseblood in the large quantities which proved necessary for completing the present study.

Dilutions of cultures for plating were made in 2 per cent Difco Neopeptone dissolved in 0.85 per cent NaCl.

Even with above precautions, some variation was observed in the mean diameter of colonies of a given strain when plated on different preparations of medium. Consequently, direct comparison of various experiments was not always possible. Duplicate plates gave, however, very consistent results. All measurements of colonies were performed after 17 hours of incubation at 37° C. Longer incubation produces larger colonies, but autolysis becomes very pronounced when incubation is continued

beyond the chosen time interval. Measurements were carried out with a Zeiss ocular screw-micrometer mounted on a binocular dissection microscope The overall magnification was 9 fold. The petri dishes were held on an inclined stage, and their surface illuminated obliquely. With an even illumination, the colony margins were very sharp. The diameter of a colony was measured at right-angles to the direction of the inclination.

The pneumococcal strains which were transformed were R36A, the rough strain used in the classical experiments of Avery, MacLeod and McCarty (2), and SIII-1-T50, an intermediate smooth strain produced by transformation of the above with an extract obtained from the mutant Type III strain SIII-1 (15).

At the outset, transforming extracts were prepared from the Type III strain A66. This strain contains the normal, or SIII-N capsular agent, and forms very large amounts of polysaccharide. As the present study evolved, transforming extracts were prepared from various clones isolated from transformation experiments. These will be described in the course of presenting the results.

EXPERIMENTAL RESULTS

1. *The diameters of SIII-N colonies of independent origin.* If the capsular agent induces transformations in an all-or-none fashion, independently transformed bacteria should be identical with respect to capsule. A definitive study of this point is not possible, owing to lack of quantitative and qualitative methods of studying polysaccharide secretion in a large sample of clones set up from such bacteria. However, a procedure was developed which, with certain reserves, can be considered to serve this purpose. Differences in the amounts of polysaccharide secreted are often reflected in differences in the diameters of colonies: the more polysaccharide, the larger the colony. Thus, measurements were made on many colonies derived from Type III pneumococci which had arisen independently of each other by transformation of an unencapsulated strain. The characteristics of the distribution obtained were compared with those of a second distribution, obtained from measurements of colonies derived from the progeny of a single Type III pneumococcus, also made by transformation. This second distribution is a control, describing the variability of the diameters attained by colonies grown from sister cells. If the capsular agent acts in an all-or-none fashion, the characteristics of these two sorts of distributions should be identical, for the variability in both instances should be due only to normal variation in the growth on plates.

The first, or experimental distribution, was obtained in the following way. The strain undergoing transformation was treated with the Type III agent under optimal transformation conditions. After incubation, dilutions of the treated cultures were spread on blood-agar plates. All colonies which were

more mucoid than the inoculated strain were measured, provided they did not fall in a crowded area. At least four treated cultures were employed for establishing a single distribution. Thus, while some of the mucoid colonies on a plate made from a given culture might be the progeny of a single transformed bacterium, the colonies measured for a single distribution cannot be derived from less than four independent transformation events. It is indeed probable that they are derived from very many more.

The addition of as little as 0.005 micrograms of DNA, isolated from an SIII-N strain, to an appropriate medium containing transformable pneumococci suffices to induce the transformation of a few bacteria. It has been found that the addition of 1000 times this amount of nucleic acid induces transformation of about 0.5 per cent of the unencapsulated bacteria during a time interval of 30 minutes, provided the bacteria are in the state which permits them to react with the nucleic acid (10). Under the conditions of the present experiments, the transforming agent is present in about 1000 fold excess of the minimal amount required to obtain transformation. At the time when the bacteria become capable of reacting with the agent, their population density is 16 000 per cc. The reactive state persists for two hours. Assuming an incidence of transformation of 0.5 per cent which is a minimum estimate, the encapsulated bacteria appearing at the end of the incubation period normally given to transformation cultures (17–20 hours) would on the average derive from 16×5, or 80, transformations in each of the treated cultures.

In the earlier experiments, treated and control cultures were incubated 18–20 hours prior to spreading on plates. However, it was found that this long incubation led to a wide spread in the measured diameters, owing, no doubt, to the onset of adverse conditions after the cessation of growth in the transformation medium. In later experiments, platings were made after only 12–14 hours of incubation. Nonetheless, the distributions of colony diameters obtained from transformation experiments are somewhat wider than those observed when the same bacteria are taken from blood-broth medium.

The clone utilized for preparing the control distribution was derived from a single Type III colony isolated from a previous transformation of the same strain upon which the experimental distribution was to be obtained. Control clones were grown under the same conditions as those employed for inducing transformations, in order to eliminate differences in the distributions which might be due to or result from differences in cultural conditions prior to plating.

The results of a typical experiment are shown in Fig. 1. In this particular instance, the strain undergoing transformation was SIII-1-T50. As has been already stated, the control clone came from a single SIII-N colony recovered

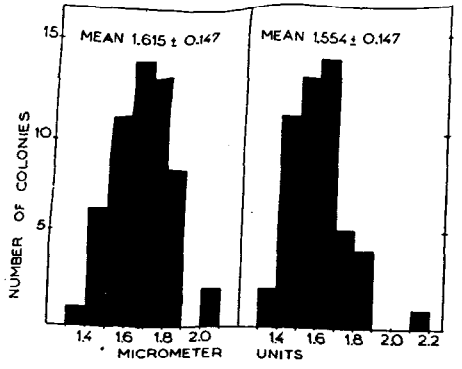


Fig. 1. Histograms constructed from the measurements of diameters of colonies of encapsulated Type III pneumococci. On the left, the bacteria came from four cultures in which multiple transformations had been induced. On the right, the bacteria came from a clone isolated a few days previously from a transformation experiment. The strain undergoing transformation was SIII-1-T50. Both the means and the standard deviations are essentially the same.

from a transformation of strain SIII-1-T50 a few days earlier. It can be seen that the distributions of the diameters of the SIII-N colonies are essentially the same whether the bacteria come from the control clone, or directly from a transformation culture. It is clear, therefore, that there is no obvious difference between the various SIII-N colonies which arise from the control clone, and those which arise from different transformed SIII-1 pneumococci. Thus, the SIII-N agent does not appear to give rise to a spectrum of pneumococci having graded polysaccharide secretion, but on the contrary seems to behave as a discrete entity.

If now new clones are set up, selecting the largest and smallest of the colonies of a particular distribution, usually, all of these clones give rise to essentially identical distributions, when the colonies formed by them are measured, and these distributions do not differ from the original one. However, the following exception to this rule is observed: from time to time, when a clone is set up from a very large colony, it gives rise to colonies which are on the average about 25 per cent larger in diameter than the majority of SIII-N colonies measured. These constitute the LC clones.

The appearance of these clones in the transformation cultures is very rare. Often, none are found. However, in one out of ten distributions obtained from transformed cultures of strain SIII-1-T50, a definite bimodality was found. This aberrant distribution is shown in Fig. 2. The second mode was found to be due to the presence of an unusually large number of LC clones in this particular experiment.

Attempts were made to determine the average number of cells per colony in the two kinds of clones. Several well isolated colonies from each were homogenized in a measured volume of medium, and serial dilutions of the suspensions plated. It was found that whereas the results were concordant when the colony came from an SIII-N-

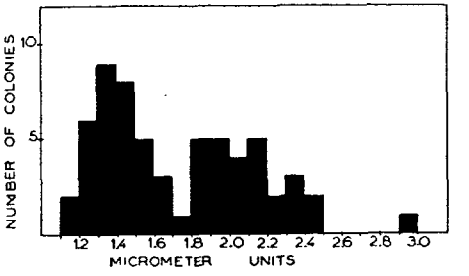


Fig. 2. Histogram constructed from measurements of encapsulated Type III colonies grown from bacteria of four cultures in which multiple transformations had been induced. Out of ten sets of such measurements, only this one showed bimodality. The peak on the right is caused by the presence of an exceptionally large number of large colonies in this particular experiment. The peak on the left corresponds to the single peak in Figure 1.

LC clone, they were aberrant when the colony was SIII-N. The aberration consisted of an excess of colonies in the higher dilutions. An experiment was set up to see whether concordant colony counts would not be obtained if the initial suspension were incubated a short time before diluting and plating. This was tried, leaving the suspensions 15 minutes at 37° C, and one hour in the refrigerator before diluting and spreading on plates. The counts on serial dilutions were then in agreement, in both types of clones. Furthermore, while the average number of cells per colony of SIII-N-LC remained unchanged by this treatment, the number of cells per SIII-N colony was increased by 30 per cent. The ratio of these averages was 3:1. The experiments are of interest in that they suggest that SIII-N colonies may be smaller because these bacteria have a lowered viability under aerobic conditions, undergoing autolysis more readily.

From these experiments it may be concluded that the SIII-N pneumococci produced by the action of the SIII-N transforming agent on strains R36A and SIII-1-T50 fall into one of two categories of which the first is more frequent: clones which form colonies of the diameter shown in Figure 1, and clones characterized by unusually large colonies. In Fig. 3 are shown the relative diameters of normal and LC clones. The data for this figure were obtained by measuring the colonies produced by one LC clone and one normal clone, grown under identical conditions and measured the same day. Fig. 3 also includes measurements made the same day on strain SIII-2, an intermediate smooth Type III clone which has been mentioned very frequently in previous publications (4, 13, 15). The three clones are quite distinct.

In the course of this study, a surprising mutability was observed in the clones derived from the transformed bacteria. In one experiment, after 3 transfers of 13 clones, two contained mutant forms which were less mucoid than SIII-N pneumococci. In another experiment, similar mutations were observed in 3 out of 8 clones after growth of the first transfers. This may be due to a real mutability of the capsular agent itself in these transformed clones. On the other hand, it may be that unencapsulated strains tend to evolve toward a state in which capsular synthesis is disadvantageous, so that endowing them with capsules by transformation upsets the pattern of selection. Under these circumstances, mutations of the capsular agent,

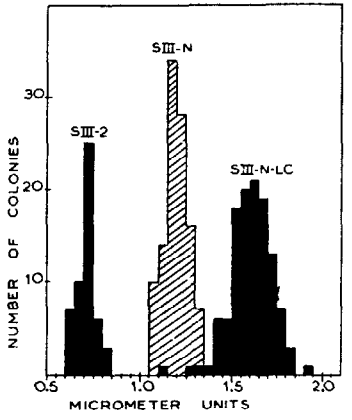


Fig. 3. Histograms constructed from measurements of colonies grown from three different clones of pneumococci. Clones SIII-N and SIII-N-LC were isolated from single colonies, the measurements of which are included in Fig. 2. Clone SIII-N came from a colony in the left hand distribution, while clone SIII-N-LC from a colony in the right hand one. For comparison, colonies of mutant Type III strain SIII-2 were measured simultaneously and included in the diagram.

leading to diminished capsule synthesis, would be favored, as would also be favored mutations reversing the trend established by prolonged existence in the unencapsulated state.

2. *The homogeneity of type III strain A66.* Since the transforming extract of strain A66 obviously induces the formation of two kinds of Type III pneumococci, it becomes essential to know whether strain A66 is heterogeneous, being composed of these two cell-types, or whether, on the contrary, both activities reside in the nucleic acid fraction of one cell-type. Therefore, measurements were made upon colonies of the stock strain A66. On the same day, and on the same media, measurements were also made on colonies of a clone of SIII-N pneumococci, isolated from a previous transformation experiment. The results of these measurements are shown in Figure 4. It is clear from these measurements that the colonies of strain A66 have a mean diameter about 20 per cent larger than that of the SIII-N colonies of the control clone. Thus, strain A66 is best described as an SIII-N-LC clone. There is no indication that the smaller colony type is present in the population A66 in any appreciable numbers.

This being so, the simplest working hypothesis consists of supposing that both colony types arise from the activities of the nucleic acid isolated from an essentially homogeneous population, and that the two activities are due to two factors which reside in each pneumococcus in the A66 population. One of these factors is the capsular agent, while the other is presumably a new agent, responsible for the large colony size. The rareness with which SIII-N-LC pneumococci are formed by transformation would thus be the consequence of the rareness of two different transformations taking place in the same bacterium.

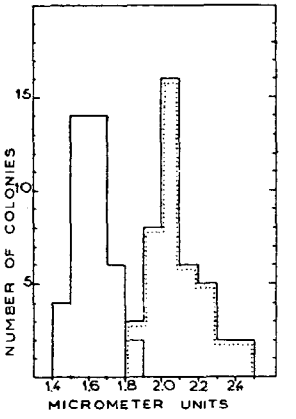


Fig. 4. Histograms constructed from measurements of colonies derived from strain A66, on the right, and an SIII-N clone on the left. The SIII-N clone was made by transformation of an SIII-1 bacterium by an extract of strain A66.

3. *The capsular agents present in SIII-N and SIII-N-LC strains.* If the above hypothesis is correct, the capsular agents in both types of clones should be the same. To test this point, nucleic acid extracts were prepared from two clones, one SIII-N-LC and one SIII-N (TPs 36E and 30B, respectively). With these extracts, transformations were induced in strains R36A and SIII-1-T50, the treated populations were spread on plates, and the colonies obtained measured in the usual fashion. In a typical experiment, 65 smooth colonies from plates of cultures treated with TP 36E and 96 from plates of cultures treated with TP 30B were measured, yielding the mean values of 2.02 ± 0.173 and 1.95 ± 0.117 micrometer units respectively. The difference between the means is not significant, and it can be concluded, therefore, that both TP preparations induce the formation of essentially the same sort of encapsulated bacteria. Thus, strain SIII-N-LC contains the same capsular agent as strain SIII-N.

However, it was noted in the course of these experiments that in the cultures treated with TP 36E there occasionally appear some SIII-N-LC clones, while none are found in the cultures treated with TP 30B. In its biological activity, TP 36E thus resembles the transforming extracts prepared from strain A66. This finding is entirely in agreement with the hypothesis that the LC trait is determined by an agent similar to the capsular agent, which can be transferred to pneumococci by the transformation technique.

4. *The isolation of unencapsulated LC clones.* If the properties of the LC strains are determined by a transforming agent, and if this agent is independent of the capsular agent, then one should expect unencapsulated bacteria to be able to acquire the former without the latter. Indeed, this transformation should occur more frequently than the double transformation in which

both are acquired. Detection of R-LC or SIII-N-LC bacteria would depend upon whether transformation by the LC agent alone suffices to produce a recognizable morphological change.

A careful search among the previously supposed "untransformed" members of treated populations of R and SIII-1 pneumococci revealed that some of the colonies produced by them were, in fact, different from ordinary R or SIII-1 colonies in that they were somewhat larger and very much more opaque. The incidence of the aberrant colonies in the treated cultures was never greater than 0.7 per cent. None were ever found in cultures which had not received a nucleic acid preparation from an LC strain. Clones derived from the aberrant colonies were established, but these could be maintained free of reversions only when carried on solid medium (blood-agar). Measurements of colonies of normal and aberrant clones of strain SIII-1-T50 gave the average values of 0.796 ± 0.143 and 0.983 ± 0.192 micrometer units. The differences between the means is not significant, and, indeed, on the basis of size alone it would be difficult to ascertain that two distinct colony types exist. However, the opacity of the aberrant colonies permits an almost certain differentiation when the plates are examined in transmitted light.

Since it was suspected that these aberrant colonies might be derived from pneumococci which had acquired the hypothetical LC agent but not the SIII-N agent, the next step was to transform them with the capsular agent, to see whether or not they yielded SIII-N-LC bacteria. Just prior to subjecting various clones to transformation, they were transferred from plates into liquid medium, using large inocula. These cultures served to inoculate transformation cultures and were discarded afterwards. In the particular experiments to be described, the aberrant clones were transformed with a nucleic acid extract of strain A66. At the same time, controls were made by transforming normal strains with the same nucleic acid preparation. The treated populations were streaked on sectors of agar medium plates, rather than being spread for measurement. From each treated population, one transformed colony was chosen at random, grown out in blood-broth medium, and samples of the resulting culture plated for measurement: Quadruplicate transformation cultures were made of each aberrant clone and of each control strain. Thus, for each strain treated with the nucleic acid fraction of strain A66, the progenies of four independently transformed bacteria were measured. This method of study was chosen to avoid the greater variability in colony diameter which is observed when measurements are made directly on the colonies of the transformation cultures themselves. The results of some measurements are shown in Fig. 5. From these observations, it is clear that when

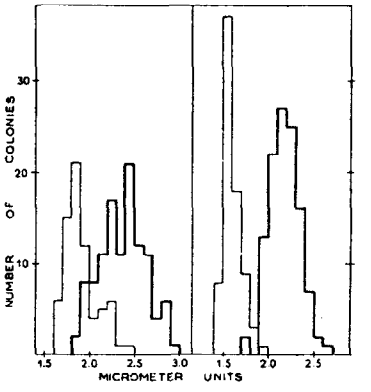


Fig. 5. Histograms constructed from measurements of SIII-N colonies derived from transformation of normal and aberrant clones of strains SIII-1-T50 and R36A. For the pair of curves on the left, the left-hand distribution came from measurements of colonies of transformed normal SIII-1 pneumococci, while the distribution on the right came from measurements of transformed aberrant SIII-1 pneumococci. The pair of distribution curves to the right are the results of similar measurements on transformed normal and aberrant R36A. In both cases, the transformed aberrant clones gave rise to larger colonies on the average than did the transformed normal clones.

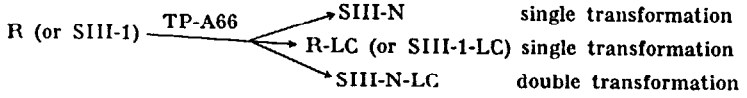
the clone undergoing transformation is an aberrant clone, be it R or SIII-1, the SIII-N bacteria induced are all SIII-N-LC; that is, they form colonies with a mean diameter roughly 25 per cent larger than that of colonies of the majority of the transformed bacteria arising in the normal strains of R or SIII-1 pneumococci. This result is entirely consistent with the hypothesis that the aberrant R and SIII-1 strains already contain the postulated LC agent, which they had acquired independently of the capsular agent in a previous transformation.

The aberrant R and SIII-1 clones will thus be referred to as R-LC and SIII-1-LC clones.

5. *A quantitative study demonstrating that the LC agent is a specific component of nucleic acid extract 36E.* Pneumococci exhibiting the LC phenotype were found only when R or SIII-1 bacteria were treated with a nucleic acid fraction prepared from strain A66, or from an SIII-N-LC strain recovered from a transformation experiment. Since in general the incidence of LC clones is low in a transformed population, it was conceivable that failure to find such clones after treatment with other nucleic acids might be due to sampling error. Accordingly, an experiment was performed in which populations of strain R36A were treated with nucleic acid extracts 36E and 30B, the former obtained from an SIII-N-LC strain and the latter from SIII-N, the incidence of R-LC colonies was scored, and the data analysed statistically. A total of 11 transformation cultures were studied, 4 receiving TP-36E and 7 receiving TP-30B. Samples of the treated cultures were spread on plates, and after incubation, the total number of colonies and the number of LC colonies per plate were estimated from counts. A large number of colonies from the seven cultures treated with TP 30B were carefully examined. After

searching for LC colonies by holding the plates against a light source, they were examined under a binocular microscope. Whereas ten LC colonies were found on the plates made from the four cultures treated with TP-36E, none were found on those made from the seven cultures treated with TP-30B (see Table I). In view of the large number of colonies examined, it is very unlikely that the observed difference is due to sampling. The experiment thus confirms the hypothesis that TP-36E contains a specific agent responsible for the appearance of the LC bacteria in the treated populations and that this agent is absent from TP-30B.

All of the biometric and transformation studies agree with the hypothesis that the nucleic acid fractions of strain A66 and of SIII-N-LC strains contain a hitherto unrecognized transforming agent which is responsible for the LC phenotype. The presence of this agent in strain A66 accounts for the size difference observed between colonies of this strain and the majority of colonies arising from transformations induced with nucleic acid derived from it, for the two transformable strains most frequently used in transformation experiments differ from strain A66 by at least two factors: the capsular agent and the LC agent. Transformations induced by nucleic acid extracts prepared either from strain A66 or from SIII-N-LC strains produced by transformation thus consist of the following inductions:



II. METABOLIC STUDIES

The inconvenience of colony diameter as a criterion for the identification of clones is that it lacks specificity. Thus, the homogeneity of the class of strains which have been called LC remained doubtful until a more specific criterion for identifying them was found. The metabolic studies to be presented below provided such a criterion.

The observed differences in colony size are manifested on blood agar plates; that is, under aerobic conditions and where glucose limits the amount of growth. A metabolic difference was therefore sought in the aerobic metabolism of glucose by normal and LC strains. In addition, a second possible difference was sought, since it had been noticed that the two types of colonies produced different degrees of greening of the blood. Since greening is due to the liberation of H_2O_2 by the bacteria, manometric experiments on glucose

oxidation were set up in such a way as to permit a simultaneous study of the amount of H_2O_2 formed by each of the strains.

No systematic study has apparently ever been made of the oxidative metabolism of pneumococci. Finkle (5) observed differences in the Q_{O_2} of three smooth races of different capsular type, as well as in rough races derived from the latter. In phosphate buffer, the optimum O_2 consumption was found to occur at pH 7.8. Observations of Sevag and Maiweg (13) demonstrated that without the addition of protective agents to the bacterial suspensions, respiration was rapidly inhibited by the accumulation of H_2O_2. Catalase and pyruvate were effective in maintaining enzymatic activity, the former by destroying peroxide, the latter by reacting chemically with it.

←TABLE I. Comparison of the transforming activities of two different transforming extracts, the one prepared from an SIII-N-LC clone (TP-36E) and the other from an SIII-N clone. The strain transformed is R36A. Four cultures were treated with the first TP and seven with the second, and from each culture the indicated number of colonies was examined.

TP	Colonies examined	LC
36E	4 456	1
	5 352	1
	5 192	2
	8 200	6
Total	23 200	10
30B	12 222	0
	17 208	0
	14 274	0
	7 770	0
	9 705	0
	9 224	0
	11 484	0
Total	81 887	0

TABLE II. Comparison of an LC and a non-LC strain with respect to ability to oxidize lactate. ↓

Strain	μ moles glucose	μ moles lactate	μ L. O_2 consumed	Moles O_2 / M. glucose
SIII-1	5	—	182	1.58
	—	10	44	
	—	—	5	
SIII-1-LC	10	—	120	0.51
	—	10	3	
	—	—	6	

Catalase added. Duration of expt. 3 hours

MATERIAL AND METHODS

1. *General method.* In order to study glucose oxidation, inactivation of enzymes by peroxide must be avoided. At the same time, to work constantly in the presence of catalase is impractical since it renders impossible study of the formation of H_2O_2. The following method was therefore adopted: thick bacterial suspensions are prepared, and a very limited amount of glucose was added. Respiration was allowed to go to completion. One Warburg vessel received glucose and catalase, and when O_2 consumption fell to the level of endogenous respiration, more glucose was tipped in to verify that the cessation of respiration had not been due to enzyme inactivation. In a second vessel catalase was held in reserve while the glucose was oxidised. When respiration ceased, the catalase was tipped in and the released oxygen measured. Since under these conditions, the difference in respiration in the presence and absence

of catalase was always equal to the amount of oxygen released by catalase in the second vessel, it is assumed that the glucose in the second vessel was completely oxidized, and that a correct evaluation of H_2O_2 production is obtained.

In some experiments, glutathion was employed for study of H_2O_2 production since this substance reacts rapidly with H_2O_2 without producing a change in gas volume (11). Glutathion as well as thioglycollic acid is very effective in protecting the activity of pneumococcal enzymes in the course of glucose oxidation, and the advantage of this medhod is that it makes it certain that oxidation in the absence of catalase has gone to completion. Two experimental vessels are required: one receiving glucose and catalase, the other glucose and glutathion, the difference in the volume changes being equal to the amount of O_2 liberated by catalase in the first vessel. At high pH values, the inconvenience of the method resides in the difficulty of correcting accurately for the autoxidation of the SH compound when it is in excess. In this respect, thioglycollic acid appeared to be the better reagent. However, with strains of bacteria in which peroxide production is known, these substances could be used very advantageously in various metabolic studies, since any appreciable excess of the SH compound could be avoided. All difficulty can be avoided, of course, by working at a pH below 7. It should be noted that the ability of SH compounds to react rapidly with H_2O_2 has been overlooked by bacteriologists and biochemists, who have been inclined to explain the beneficial action of these substances on the growth of certain microaerophilic bacteria as due to a direct action on the SH groups of bacterial enzymes. It is likely that in certain cases their primary activity is simply to destroy hydrogen peroxide.

2. *Pneumococcal strains.* Various LC and non-LC strains were studied. However, the bulk of the work was done on two clones, one normal, and one LC, both of which possessed the mutated capsular transforming agent SIII-1. Since LC strains are stable only on solid medium, both normal and LC clones were carried on agar to avoid any differences in cultivation of the strains to be compared.

3. *Preparation of cell suspensions.* Pneumococci undergo autolysis readily, so that great care must be exercised in growing the bacteria and preparing cell suspensions. All cultures were initiated by transferring several loopfuls of cells from agar petri dish cultures to liquid medium (medium 1, reference 4). After the first appearance of turbidity, the cultures were incubated another 2 hours and placed in the refrigerator for the night. Next morning, after addition of 0.5 cc of a sterile 2.5 per cent glucose solution per 100 cc of culture, the cultures were placed for 1 hour, in a 37° C water bath. Thereafter, the bacteria were harvested by centrifugation, washed once with a small volume of the salt mixture to be described below, and resuspended in the desired volume of this same solution. 200 cc of culture sufficed for a 4 vessel experiment, the washed cells being taken up in 9 cc of salt solution. For larger experiments, larger amounts were prepared in the same way. Suspension density was controlled photometrically. Microscopic examination revealed that suspensions contained short chains of gram positive diplococci, homogeneous in size.

4. *Solutions.* All solutions were prepared in Pyrex distilled water. The suspension medium contained 42 mg of NaCl, 230 mg of KCl, 60 mg of CaCl and 95 mg of $MgSO_4 \cdot 7H_2O$ dissolved in 75 cc of water. To this were added 25 cc of M/7.5 phosphate buffer pH 7.8.

Warburg vessels receiving catalase were given 0.2 cc of a solution of crude catalase powder, 1/1000 (Armour and Co.) prepared fresh for each experiment.

The glucose employed was "Dextrosol" (Société des Produits du Maïs, Paris), and the lactate a racemic preparation (Malinkrodt).

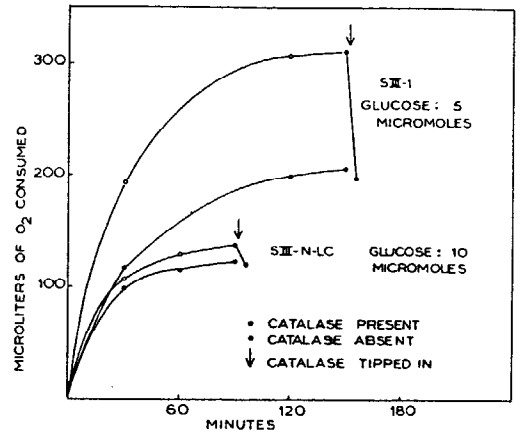


Fig. 6. Results of a typical respiration experiment with an LC strain and a non-LC strain of *Pneumococcus*.

EXPERIMENTAL RESULTS

1. *Respiration and H_2O_2 formation with glucose.* The results of a typical experiment on a normal and an LC strain are shown in Fig. 6. In this particular instance, the non-LC strain contained a mutated capsular agent, while the LC strain contained a normal capsular agent. As will be shown presently, the kind of capsular agent possessed by the bacterium has no influence on its respiration in the presence of glucose. From this figure, two differences between LC and non-LC strains can be seen. First, the LC strain consumes very much less oxygen per molecule of glucose than the non-LC strain. Second, H_2O_2 production by the LC strain is both absolutely and relatively less than that of the non-LC strain.

For purposes of comparison of the respiration of various strains in the presence of the same amounts of glucose, Fig. 7 has been prepared. It summarizes results obtained with three kinds of strains: *a*), non-LC strains, represented by R36A, SIII-1-T50, and an SIII-N strain made by transformation of the latter with the nucleic acid extract of strain A66, and in the course of which only the SIII-N capsular agent was acquired; *b*) an LC strain, made by transformation of strain SIII-1-T50 with the above nucleic acid extract, and as a result of which both the LC agent and the capsular agent were acquired: and *c*), strain A66 itself, the stock laboratory strain which

possesses both the SIII-N capsular agent and the LC agent. For each molecule of glucose, the first group of strains consumes 1.6–1.8 molecules of oxygen, the second strain, about 0.4 molecules, while the last strain consumes 1.1–1.2 molecules. In each instance the cessation of oxygen uptake was due to exhaustion of glucose.

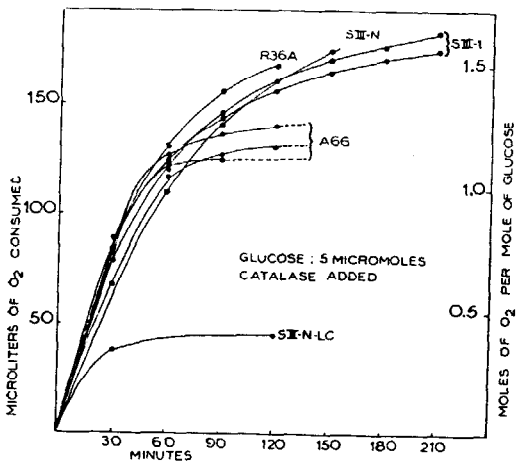


Fig. 7. Respiration of various strains of *Pneumococcus* in the presence of 5 micromoles of glucose. Strains R36A, SIII-1 and SIII-N are non-LC strains; Strain A66 is the naturally occurring Type III strain in which the LC agent was first found; Strain SIII-N-LC is and LC strain made by transformation of strain SIII-1-T50 with an extract from strain A66.

Two points of interest can be noted in Fig. 7. First, particularly evident is the magnitude of the difference between the non-LC strains and the LC strain made by transformation. Second, it is quite clear that although strain A66 has a less active oxygen consumption than the non-LC strains, it is nonetheless more active than the LC strain made by transformation. Thus, although strain SIII-N-LC has acquired two agents from the nucleic acid fraction of strain A66, the two strains are not identical in their metabolic properties. It must be supposed, therefore, that strain A66 differs from strains R36A and SIII-1 not only in possessing the LC and SIII-N agents, but also in an inherited ability to carry out further oxidation of glucose in spite of the LC agent being part of its genetic constitution.

Although strains A66 and SIII-N-LC differ in this respect quantitatively, qualitatively their respiration is very similar. Strain A66 forms very little free hydrogen peroxide, just as strain SIII-N-LC. An even more striking qualitative similarity between strain A66 and LC strains made by transformation will be described in the section below.

2. *Respiration in the presence of other substrates.* Neither LC or non-LC pneumococci, grown as described above, oxidize pyruvate, gluconolactone or 6-phosphogluconate. However, with respect to oxidation of lactate, the

two kinds of pneumococci differ sharply. Whereas lactate is oxidized by strains R36A, SIII-1-T50 and by most encapsulated strains produced by their transformation, this substrate is non oxidized by strain A66, or by any LC strain produced in the transformations described above. Typical experimental results are shown in Table II. Again, it should be emphasized that ability

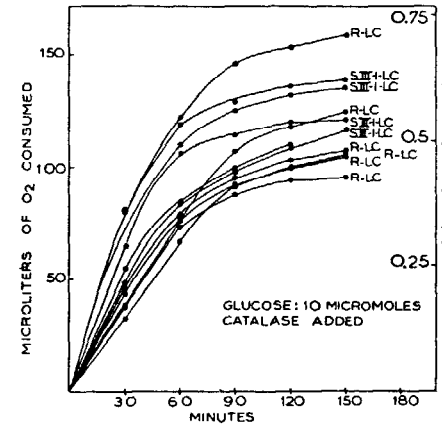


Fig. 8. The respiration of various LC strains of independent origin. Each strain is the product of an independent transformation induced by the LC agent. The variation in the amount of oxygen consumed per molecule of glucose is no greater between these strains than that observed in independent measurements on a single strain.

to oxidize lactate is independent of the capsular constitution of the strain studied, and is strictly correlated with the presence of the LC agent.

3. *Metabolic study of various newly derived LC strains.* With these specific criteria for identifying LC strains at hand, there remained to be established whether LC clones derived from independent transformations, and identified morphologically, constituted a homogeneous physiological class. Therefore, many LC clones were studied manometrically. The clones were obtained from various sorts of transformations: R to R-LC, SIII-1 to SIII-1-LC, and SIII-1 to SIII-N-LC. The inducing agents came from strains A66, 36E and 30A. Strain A66 is the natural source of the LC agent, strain 36E is an SIII-N-LC strain produced by a double transformation of strain SIII-1-T50, and strain 30A is an SIII-N-LC strain produced by single transformation of an SIII-1-LC strain.

In every instance, a clone which had been identified on morphological grounds as LC, proved to have the metabolic characteristics of the LC strains described in the preceding two sections. For example, every LC clone isolated from the experiment shown in Table I was tested manometrically, and proved to have metabolic properties characteristic of LC strains.

Fig. 8 shows a plot of the measurements made on 11 different LC strains of independent origin. The amount of oxygen consumed per molecule of

glucose varied from 0.4 to 0.75, but in no case approached the value of 1.6, characteristic of the non-LC strains. The variation between strains, shown in Fig. 8, is no greater than that observed with a single clone on different days. Many of these strains were also studied with respect to ability to oxidize lactate, and proved not to respire it.

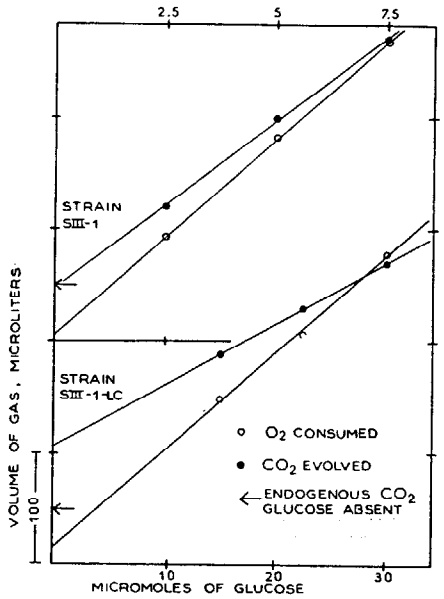


Fig. 9. The determination of respiratory quotients of LC and non-LC strains in the presence of different quantities of glucose. The LC strain has a pronounced endogenous CO_2 production which takes place only when glucose is added. The RQ values for the lower pair of lines are 0.58, 0.62, and 0.61; for the upper pair of lines, 0.74, 0.82, and 0.82.

The experiments demonstrate, therefore, that those clones which have been called LC because of the characteristics of their colonies constitute a distinct and homogeneous group with respect to their metabolic properties. Since the presence or absence of a capsule in no way influences these properties, we can say that the LC agent exhibits a complete physiological autonomy with respect to the capsular transforming agent.

4. *Respiratory quotients of strain A66, and LC and non-LC strains.* Respiratory quotients were determined on various of the strains described above, in order to further characterize them. Strains A66 and SIII-1-T50, when studied by the direct method, gave very reproducible values, 0.95 and 0.8 respectively. However, an LC strain derived from SIII-1-T50 proved exceedingly variable, until it was found that endogenous CO_2 production was not the same in the presence and in the absence of glucose. The technique was then adopted of running three different concentrations of glucose simultaneously, and extrapolating for both endogenous respiration and CO_2 production.

Values ranging from 0.5 to 0.6 were then obtained. Fig. 9 shows the results of such an experiment.

The RQ of strain A66 is typical for lactic bacteria. The RQs of strain SIII-1-T50 and the LC strain derived from it are not. A more striking deviation from the classical picture of respiration is the high endogenous CO_2 production by the LC strain, which takes place only in the presence of glucose.

5. *The formation of lactic acid from glucose by an LC strain.* It was pointed out to me by Dr. H. Kalckar that since LC strains do not oxidize lactate, they might also fail to form lactic acid by fermentation of glucose. An experiment was therefore devised to test this point. An LC strain, derived from transformation of strain SIII-1-T50, was seeded simultaneously into two 300 cc erlenmeyer flasks, the one containing 200 cc of basal medium (medium 1, reference 4), and the other containing the same medium to which a measured amount of glucose was added. These cultures were incubated for two hours after appearance of turbidity, and placed in the refrigerator until the next day. The bacteria were then removed by centrifugation (1 hour), and the supernatant media decanted carefully. A portion of each supernatant liquid was treated with $CuSO_4$ and $Ca(OH)_2$ to remove interfering substance, and the amount of lactic acid determined in each by the method of Friedmann and Kendall. The difference in lactic acid content of the two culture media

TABLE III

Determination of the amount of lactic acid formed by strain SIII-1-LC from a given amount of glucose. The basal medium contained an unknown amount of lactic acid and substances capable of giving rise to lactic acid in the course of bacterial growth. Lactic acid present after growth was compared in basal medium, and in medium to which a known amount of glucose was added at the time of inoculation.

Solution analysed	mg lactate expected	mg lactate found	Per cent recovery
Calcium lactate	1.22	1.18	97
		1.19	97.7
Supernatant liquid, Basal medium	x	2.26	
		2.07	
		2.35	
		Av. 2.23	
Supernatant liquid, Basal Medium plus glucose	x + 1.75[1]	3.485	
		3.63	
		Av. 3.58	77

[1] Assuming that 1 molecule of glucose gives rise to 2 of lactic acid.

is presumed to be equal to the amount of lactic acid formed from the added glucose, in the course of bacterial growth. This procedure was adopted since the basal medium itself is of unknown composition. Table III shows the results of the single experiment performed. The difference in the lactic acid contents of the two media indicates that 77 per cent of the added glucose was recovered as lactic acid at the end of growth. Thus, judging from this one experiment, the conversion of glucose to lactic acid by an LC strain growing in unaerated medium follows the classical picture first described by Hewett (6, 7) for pneumococci and streptococci. The result indicates, consequently, that formation of lactic acid from glucose by glucolyses is essentially normal in the LC strain studied.

DISCUSSION

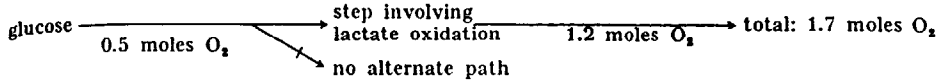
The present study has revealed the presence of a new transforming agent in the desoxyribonucleic acid of the Type III pneumococcal strain A66. This brings to four the number of naturally occurring agents which can be detected in this material: the Type III capsular agent (2), the rough agent (14), the M protein agent (1), and the LC agent. One more element of what must be an exceedingly complex mixture has thus been identified.

The LC agent could be identified owing to two fortunate circumstances. First, the unencapsulated strains usually employed in transformation studies happen to lack the agent, while the Type III strain from which transforming extracts are customarily prepared possesses it. Second, acquistion of the LC agent by non-LC pneumococci is accompanied by a slight, but unmistakable change in colony morphology.

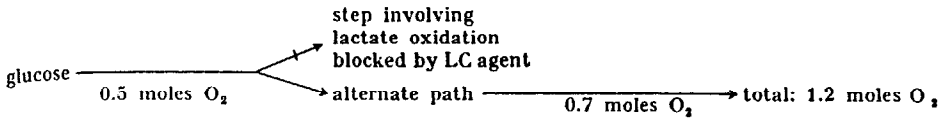
It was possible to show that in addition to altering colony morphology, the LC agent profoundly changes the metabolic pattern of the pneumococci which acquire it through transformation, for such pneumococci lose entirely the capacity to oxidize lactate, and manifest a very reduced respiration of glucose. The LC agent is the first transforming factor whose action has been shown to affect catabolic reactions.

It is not possible from the data presented here to propose a detailed hypothesis to describe the oxidative pathways in the various strains studied. In a general way, however, the results obtained can be drawn together into a coherent scheme on the assumption that the initial steps of glucose oxidation are identical in all strains studied, and that the strains which have been transformed by the LC agent can perform only this initial oxidation step. One then obtains:

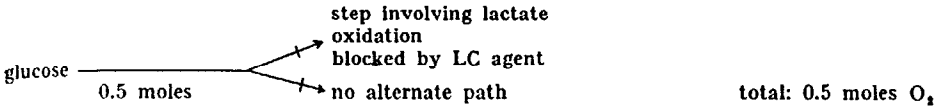
a) Pattern common to strains R36A and SIII-1-T50, and encapsulated strains derived from them by transformation, except LC strains.



b) Pattern in Type III strain A66.



c) Pattern common to strains of type *a* which have acquired the LC agent.



The CO_2 released in the three kinds of oxidations is respectively 1.35, 1.1 and 0.25 molecules per molecule of glucose. Thus, assuming again that the residual respiration of strains of type *c* is the first respiration step, common to all strains, the RQ of the oxidation beyond the first step is 1.1/1.2 for type *a*, and 0.58/0.7 for type *b*. It should be added that the values found for O_2 uptake were variable in strains of type *c*, ranging from 0.3 to 0.7, usually falling between 0.4 and 0.5 molecules per molecule of glucose. This suggests a marked influence of endogenous factors on respiration of these strains. In this connection it should be recalled that in type *c* strains, the addition of glucose stimulates the formation of CO_2 of endogenous origin, indicating very clearly the existence of important endogenous factors.

In all strains studied, the amount of H_2O_2 recovered was found to be less than would have been expected if all hydrogen transfer to oxygen gave rise to hydrogen peroxide. Strains with respiration of type *a* accumulate enough H_2O_2 in the vessel to permit one to say that 70–80 per cent of respiration gives rise to peroxide. Strains with respiration of types *b* and *c* give rise to enough free hydrogen peroxide to account for only 30–40 per cent of the respiration producing peroxide. This is a surprising result if it is true that no catalase or peroxidase activity is present in pneumococci, and that all respiration proceeds *via* flavoprotein enzymes. Recently, evidence has been obtained for a peroxidase-like activity in *Streptococcus faecalis*, as a consequence of which lactate is oxidized by hydrogen peroxide, giving rise to acetate, CO_2 and water. This reaction was shown to be enzymatic (3).

Such a reaction was sought for in the pneumococcal strains studied here, but no evidence could be found for its existence. In the course of these experiments, it was seen that when 16 micromoles of H_2O_2 are added to pneumococcal suspensions, none of it disappears in the course of two hours at 28° C. This suggests that destruction of peroxide by non-enzymatic reactions cannot account for the low recoveries of hydrogen peroxide in the respiration experiments.

A last point to be mentioned is the observation that an LC strain which fails to oxidize lactate does nevertheless produce lactic acid from glucose in the course of growth in non-aerated cultures. This is of some interest, for it has been generally supposed that the metabolism of lactate is mediated by a single enzyme, lactic dehydrogenase, which can either initiate hydrogen transfer from lactate to oxygen, or cause lactate to be synthesized through the reduction of pyruvate (see Ochoa (11), for a review of the various origins of lactate in intermediary metabolism). The characteristics of the LC strains studied here permit one to suppose that lactate arises through the activity of an enzyme which is incapable of mediating its oxidation. Thus, in those strains which do oxidize lactate, two independent enzymes may be involved. On the other hand, one might also suppose that the block introduced by the LC agent affects solely the transfer of hydrogen to oxygen, without affecting the other roles of lactic dehydrogenase, and thus avoid the necessity of postulating a second lactate-activating enzyme.

The finding that lactic acid is formed in normal amounts in the course of growth of an LC strain in glucose-containing medium is of interest for still another reason. One might have supposed that the LC agent blocks glucose metabolism at a very early stage, and that the enzyme (s) for oxidizing lactate fail to be synthetized owing to the absence of lactic acid to serve as an adaptive substrate. However, this cannot be the case since lactate is recovered from the growth medium in amounts typical for homolactic fermenting organism, and presumably is accumulated during growth.

Since both rough and Type III smooth strains have been cultured for many years under essentially identical conditions, it is somewhat surprising to find such a striking difference between them with respect to glucose and lactic acid metabolism. It could, of course, be supposed that the presence or absence of a capsule plays a critical role in determining which kind of metabolism is most advantageous. However, the answer is not so simple. The rough strain R36A was derived originally from an encapsulated Type II strain, D39. Examination of D39 was not undertaken in detail, and a few experiments performed with it have not been included in the experimental

section of the present report. Nevertheless, the data which were obtained showed that strain D39 resembles R36A in that it, too, oxidizes lactate. However, it differs from the rough strain and resembles the Type III strain A66 with respect to the quantity of O_2 consumed per molecule of glucose. Therefore, the loss of a capsule may, at best, have favored the selection of a pneumococcal variant possessing an increased ability to oxidize glucose. One is still faced with explaining how strains cultivated under identical conditions can differ with respect to ability to oxidize lactic acid. At the cellular level, no interference seems to exist between respiratory pattern and the secretion of either Type II or Type III capsule, for it was possible to make by transformation Type II strains which did not oxidize lactate, just as it was possible to make Type III strains which did. The relative selective values of the different combinations of characters were not studied, however, and it is still possible that the type of capsule secreted by a given cell plays an important role in stabilizing in populations a particular kind of respiration pattern. It is more likely, however, that other factors acting in the course of time have brought about the stabilization of different metabolic patterns in the various naturally occurring lines of pneumococci.

SUMMARY

Evidence was obtained which indicates that the normal Type III capsular transforming agent of pneumococcus acts in an all-or-none fashion, at least in the majority of transformations which it induces. No indication could be found to suggest that these inductions produce a spectrum of bacteria having graded abilities to secrete polysaccharide.

In the course of studying the transformations induced by this agent, a new transforming agent was found to exist side by side with the capsular agent in transforming extracts prepared from Type III strain A66. The new agent was detected because the rough strain used in transformation studies lacks this agent.

The new agent is called the LC (large colony) agent because its most obvious manifestation is to increase the size of the colonies formed on petri dishes by pneumococci containing it.

In transformations, the LC agent is usually acquired independently of the capsular agent. Very rarely, both capsular and LC agents are acquired by a single bacterium, in the course of a single treatment of a culture with the transforming extract.

Metabolic studies show that pneumococci which possess the LC agent have

a diminished ability to respire in the presence of glucose, and fail to oxidize lactate. Furthermore, they produce less hydrogen peroxide in the course of glucose oxidation than do pneumococci which do not possess this agent.

The metabolic characteristics of LC and non-LC strains are uninfluenced by the kind of capsule which they possess (Type III, normal or mutant, and Type II).

Applying metabolic criteria to the study of many clones derived independently by transformations with the LC agent and identified on morphological grounds, it is found that these constitute a homogeneous class with respect to the metabolic properties examined.

The LC agent is thus responsible for a particular pattern of metabolism, perhaps produced by the introduction of a block in the oxidation of lactic acid.

A typical LC strain produced by transformation was found to have the following additional characteristics: a) a pronounced formation of CO_2 from endogenous sources which takes place only in the presence of added glucose; b) an RQ atypical of lactic acid bacteria; and c) the ability to form enough lactic acid from glucose in the course of growth in unaerated cultures to account for about 77 per cent of the carbon of the added glucose, this in spite of being unable to attack lactate oxidatively under the conditions of the Warburg experiments.

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